A Novel One Step Real Time RT-PCR Assay for the Detection of Enterovirus

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Introduction

Enteroviruses (EV) are the leading cause of aseptic meningitis in pediatric and adult populations and can be associated with severe disease such as myocarditis, encephalitis, and paralytic poliomyelitis. RT-PCR has rapidly become the diagnostic methodology of choice due to its sensitivity and rapid turn-around-time allowing significant improvement in patient care and management. Most molecular assays target the highly conserved regions within the 5' nontranslated region (NTR) described by Rotbart el al. We describe the development of a novel real time assay that amplifies and detects a conserved region upstream of the Rotbart amplicon utilizing primers and an Eclipse probe. As a further enhancement, an RNA internal control (IC) is integrated into the reaction. The analytical sensitivity was determined and a comparison to the Chemicon Oligodetect Pan-enterovirus kit was made during the 2005 enterovirus season.

Results

Analytical sensitivity was determined using 2-fold serial dilutions of positive control material. The real time assay demonstrated 100% analytical sensitivity compared to the PCR-Chemicon assay (Figure 1 & Table 2).

Twenty-two of the 76 specimens were positive for enterovirus using the PCR-ELISA method, whereas the real-time assay identified only 16 out of these 22. The remaining 54 specimens were negative by both assays (Table 3).

DNA sequencing was performed on the 6 discrepant patient specimens that failed to exhibit a recognizable crossing threshold in the real time assay. Sequencing results indicated the upstream priming region in 5 of the 6 discrepant samples contained one variable nucleotide position with two distinct single nucleotide polymorphisms (SNP). Four discrepant samples contained a T to G SNP. One of the remaining two samples contained at T to A polymorphisms (Figure 2). These SNPs appear to be newly discovered during this study.

Conclusion

This real time assay design containing an RNA internal control demonstrated excellent analytical sensitivity when compared to the PCR-ELISA assay. However, upon testing individual and unique clinical samples it became evident that the clinical sensitivity was substandard due to unidentified polymorphisms underneath a primer. Although the frequency of these SNPs in nature is uncertain, the identification of 6 out of 22 (27%) positive patient specimens in this study suggests that this region may be potentially compromised in a considerable percentage of enterovirus specimens. This discovery lends a warning to all groups using or developing new assays for enterovirus that there may be additional unknown SNPs within the "conserved" 5' NTR and that these tests should be rigorously validated using clinical samples. Future directions for this assay include incorporating modified bases to accommodate the newly described SNPs.

Primers and probes	Sequence	Nucleotide positions[a]
PCR-ELISA		
EV1	5' - CCTCCGGCCCCTGAATGCGGCTAAT - 3'	449-473
EV2	5' - biotin-ATTGTCACCATAAGCAGCCA - 3'	583-602
Probe (Antisense)	5' - GAAACACGGACACCCAAAGTA - 3'	547-567
Real time RT-PCR Assay		
EV-F	5' - GA*AGAGA*CTAG*TGA*GCTA - 3'	422-439
EV-R	5' - GTTAGGA*TTAGCCGCATTC - 3'	461-479
Probe	5' - MGB - TCCGGCCCCTGAATGC - FAM - 3'	451-466
IC - Forward	5' - CCA*TCAAA*GTCGA*GGTGCCTAAAGTG - 3'	1513-1538[b]
IC - Reverse	5' - ACGAACGCCATGCGGCTACAGGAAGCTC - 3'	1563-1590
IC Probe	5' - MGB - TGTTGGTGGTGTAGAGC - PY - 3'	1550-1566

[a]Corresponding nucleotide position in Cox B5
[b]Corresponding nucleotide position in ms2 Phage
A*, G* = super A base, super G Nanogen/Epoch Biosciences

Table 1. Primers and probes used for the detection of Enterovirus from patient specimens by the 2 assays.

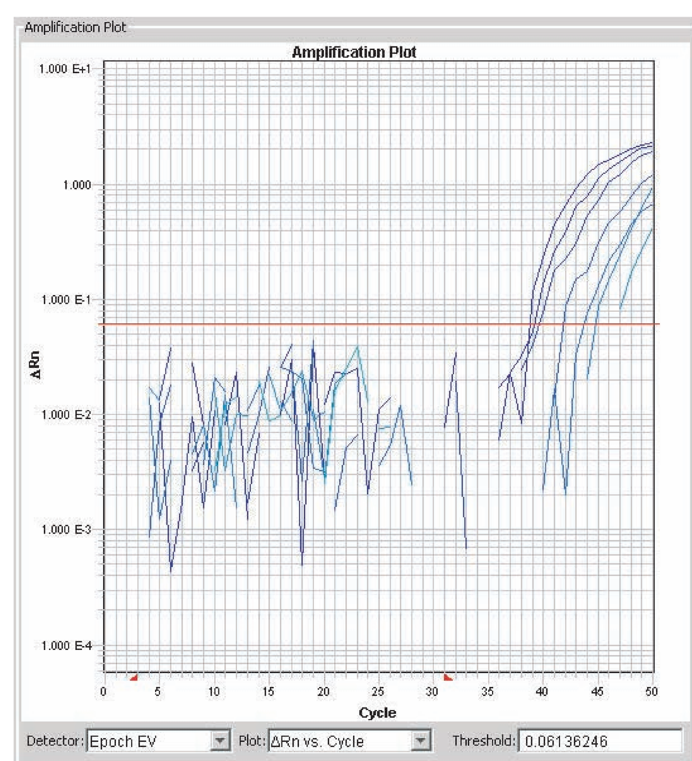

Figure 1. Amplification of 2-fold serial dilutions of positive control material.

Dilution	Real Time C_T	Chemicon OD
1	38.47	> 3.0
2	38.85	2.465
3	39.30	1.353
4	41.57	0.615
5	43.78	0.394
6	44.68	Neg
7	46.74	Neg
8	Neg	Neg

Table 2. Analytical sensitivity comparison between the 2 assays using serial 2-fold dilutions of control material.

	Chemicon +	Chemicon -
Real Time +	16	0
Real Time -	6	54

Table 3. Comparative results of the 76 samples tested in both assays .

Materials and Methods

Clinical specimens

Seventy-six patient specimens submitted for enterovirus testing at ARUP laboratories were analyzed concurrently with the PCR-ELISA and real time assays. RNA was extracted from 70 μL of each sample using the Qiagen Biorobot 9604. The RNA IC was added to the lysis buffer prior to extraction to yield a final concentration of 300 copies/reaction. All specimens were extracted in duplicate.

PCR-ELISA

RT-PCR was performed with 20 μL RNA using the OneStep RT-PCR kit and primers and probes listed in Table 1 followed by detection with the Chemicon Pan-Enterovirus Oligodetect kit per the manufacturer's instructions. RT-PCR was performed using the following parameters:

20°C - 10 minutes
50°C - 30 minutes
95°C - 15 minutes
$\left.\begin{array}{l}\text{94°C - 15 seconds}\\\text{58°C - 30 seconds}\\\text{72°C - 30 seconds}\end{array}\right\}$ 40 Cycles
72°C - 10 minutes

Real-time RT-PCR assay

Viral RNA was amplified using the OneStep RT-PCR Kit and primers and probes listed in Table 1. Each 50 μL reaction contained 20 μL of RNA, 120 nM ROX, 1.0 μM each EV primer, and 200 nM of EV probe. The IC primers and probes were at 200 nM and 50 nM respectively. The EV probe contained a 5' minor groove binder and a FAM label on the 3' end, while the IC probe was labeled with PY.

Real time RT-PCR was performed on the Applied Biosystems HT7900 using the following parameters:

20°C - 10 minutes
50°C - 30 minutes
95°C - 15 minutes
$\left.\begin{array}{l}\text{95°C - 15 seconds}\\\text{56°C - 30 seconds}\\\text{76°C - 30 seconds}\end{array}\right\}$ 50 Cycles
$\left.\begin{array}{l}\text{95°C - 15 seconds}\\\text{45°C - 15 seconds}\\\text{95°C - 15 seconds}\end{array}\right\}$ Melt

The final ramp rate of the melt was set at 5%. Data acquisition occurred during the annealing step of each cycle and during the final ramp portion of the melt.

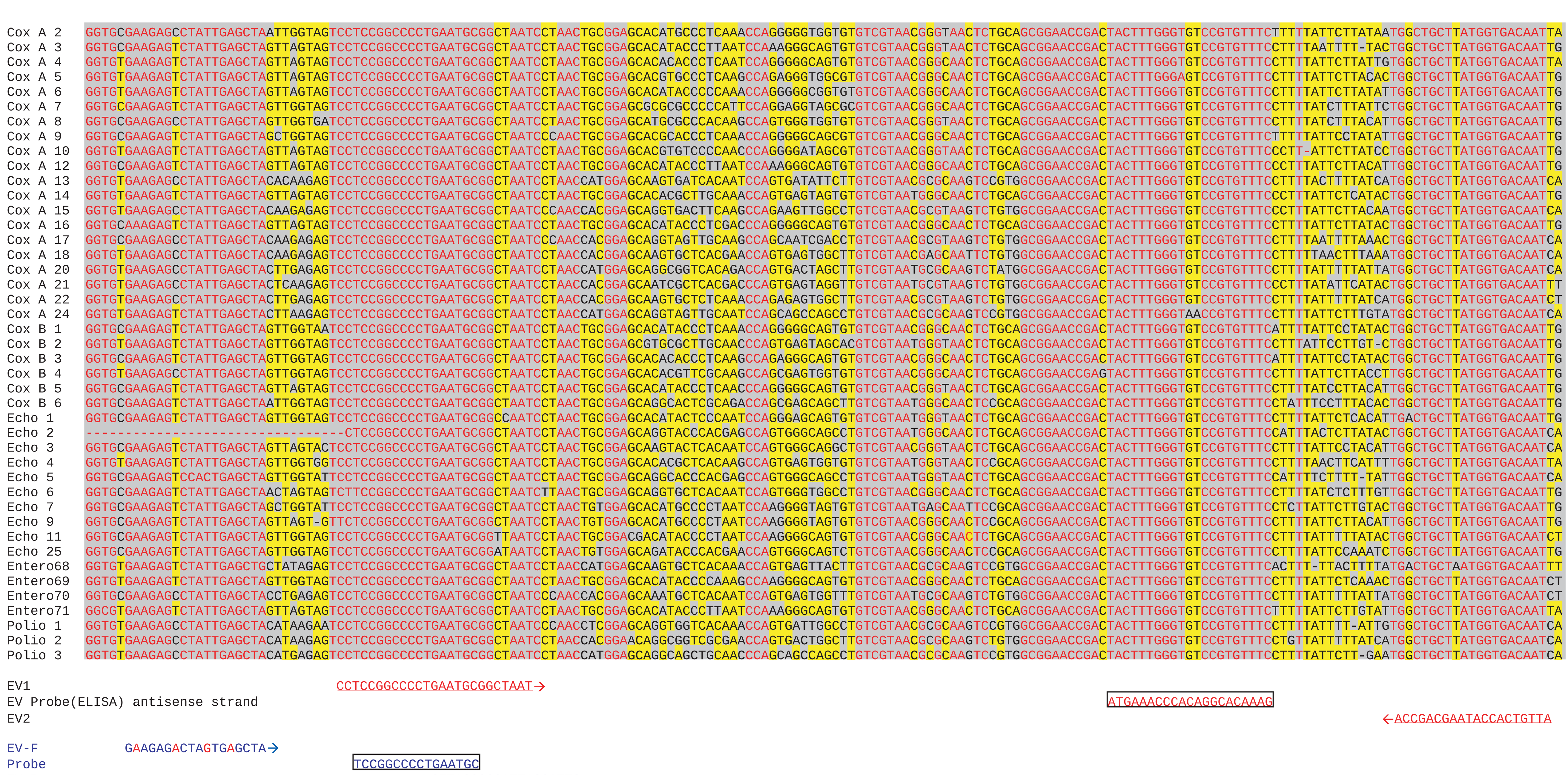

Figure 2. Electropherograms showing newly identifited polymorphisms. The upppermost electropherogram is the wild type.

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